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ORIGINAL PAPER

Involvement of reactive oxygen species during early stages of ectomycorrhiza establishment between *Castanea sativa* and *Pisolithus tinctorius*

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Abstract Evidence for the participation of reactive oxygen species (ROS) and antioxidant systems in ectomycorrhizal (ECM) establishment is lacking. In this paper, we evaluated ROS production and the activities of superoxide dismutase (SOD) and catalase (CAT) during the early contact of the ECM fungus *Pisolithus tinctorius* with the roots of *Castanea sativa* (chestnut tree). Roots were placed in contact with *P. tinctorius* mycelia, and ROS production was evaluated by determining the levels of H_2O_2 and $O_2^{\cdot-}$ during the early stages of fungal contact. Three peaks of H_2O_2 production were detected, the first two coinciding with $O_2^{\cdot-}$ bursts. The first H_2O_2 production peak coincided with an increase in SOD activity, whereas CAT activity seemed to be implicated in H_2O_2 scavenging. *P. tinctorius* growth was evaluated in the presence of *P. tinctorius*-elicited *C. sativa* crude extracts prepared during the early stages of fungal contact. Differential hyphal growth that matched the H_2O_2 production profile with a delay was detected. The result suggests that during the early stages of ECM establishment, H_2O_2 results from an inhibition of ROS-scavenging enzymes and plays a role in signalling during symbiotic establishment.

Keywords *Castanea sativa* · *Pisolithus tinctorius* · Ectomycorrhiza (ECM) · Reactive oxygen species · Catalase (CAT) · Superoxide dismutase (SOD)

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Introduction

During ectomycorrhiza (ECM) establishment, the mycobiont has the ability to recognize and become associated with host roots. In addition, the fungal symbiont must be able to escape host defence surveillance and establish bi-directional nutrient transfers across the root-fungus interface. Thus, the establishment of this symbiotic association requires a sequence of highly regulated and coordinated events, initiated by an exchange of specific signalling compounds between both partners (Martin et al. 2001; Podila 2002). The mechanisms triggering the interaction and progression of the colonization process are still poorly understood.

In plant-pathogen compatible interactions, a fungal pathogen either eludes or suppresses recognition, and plant disease ensues. In contrast, in incompatible host interactions as well as in non-host interactions, plants respond to pathogen infection by activating mechanisms of disease resistance, and consequently, no disease symptoms develop (Dixon and Lamb 1990; Kapulnik et al. 1996; Mysore and Ryu 2004). In most cases, defence responses against fungal pathogens include (1) the production of secondary metabolites, such as antimicrobial phytoalexins; (2) plant cell wall modifications via the deposition of lignin, callose, and hydroxyproline-rich glycoproteins; (3) a hypersensitive response (HR) characterized by rapid, localized, chemical defences and cell necrosis

surrounding the infection site; (4) the expression of pathogenesis-related (PR) proteins, such as chitinases, peroxidases (POXs), glucanases, and phenylpropanoid metabolism enzymes; and (5) the production of reactive oxygen species (ROS) in the so-called oxidative burst (Baker and Orlandi 1995; Lamb and Dixon 1997; Wojtaszek 1997).

During the early stages of fungal colonization, increasing evidence suggests that the plant's defence system is similarly induced in mycorrhizas, but is suppressed in a well-developed symbiotic interaction (Martin et al. 2001; García-Garrido and Ocampo 2002; Lum and Hirsch 2003). In arbuscular mycorrhizal (AM) associations, the accumulation of ROS and the induction of ROS-scavenging enzymes, such as superoxide dismutase (SOD), catalase (CAT), and POX, have been reported (Arines et al. 1994; Salzer et al. 1999; Blilou et al. 2000; Lambais et al. 2003; Fester and Hause 2005). In contrast, during the development of ECM symbioses, data detailing the involvement of the antioxidative system are still scarce. No direct evidence has been reported concerning an oxidative burst in response to ECM fungal contact with roots. Only the treatment of *Picea abies* (L.) Karst. suspended cells with elicitors released from the ECM fungi *Amanita muscaria* (L.) Lam., and *Hebeloma crustuliniforme* (Bull.) Quél. has suggested a rapid transient production of ROS, mainly H_2O_2 (Schwacke and Hager 1992; Salzer et al. 1996). The differential gene expression analyses performed during development of an ECM association have reinforced the hypothesis that an increase in plant defence/stress responses may take place (Voiblet et al. 2001; Johansson et al. 2004; Duplessis et al. 2005). In these studies, it was shown that pathogenesis-related genes and other stress-related genes (e.g. encoding metallothioneins or heat-shock proteins) are up-regulated, but no changes in transcript levels of oxidative stress enzyme genes that could account for a transient increase in ROS were observed. This result could be, in part, related to the subtle changes in the gene expression of the partners when considering the entire ECM and not the individual cells directly involved in the symbiosis (Wiemken and Boller 2002).

In this study, we have focussed on the involvement of ROS during the early contact events of mycorrhizal formation in *Castanea sativa* Mill. roots, using the model ECM fungus *Pisolithus tinctorius* (Pers.) Coker & Couch that forms symbiotic associations with many tree species (Cairney and Chambers 1997).

Materials and methods

Biological material and inoculation

P. tinctorius (Pers.) Coker & Couch (isolated 289/Marx) was obtained from the University of Tübingen and was maintained on agar Melin–Norkans (MMN) media (Marx 1969). Before the aseptic establishment of ECM, hyphal plugs of *P. tinctorius* were transferred to 250 ml of modified MMN liquid media supplemented with 5 g l^{-1} of glucose without malt extract and casamino acids (Martins et al. 1996). Fungal cultures were maintained in the dark at 25°C without agitation until a dense mycelium was observed.

Seeds of *C. sativa* Mill. (obtained in the Bragança region orchards) were surface disinfected with sodium hypochloride (5%, v/v) for 1 h and rinsed with sterile distilled water. The seeds were aseptically transferred to sterilized sand and maintained in the dark at 5–10°C for 2 months. After germination, the tips of the radicles (approximately 4 cm in length) were removed to promote root ramification, and the seedlings were placed on a non-submerged plastic net introduced into sterile flasks containing 250 ml of tap water. The flasks were maintained in a growth chamber at 19°C in the dark until leaf emergence. They were then kept under a 16-h light period (light intensity of approximately 100 mE $m^{-2}s^{-1}$) at 25°C during the light period and 19°C during the dark period for 2 months. When plants had a well-developed root system, inoculation with *P. tinctorius* was performed by transferring previously washed mycelium (4.0 g FW per flask). After gentle shaking, the flasks were maintained in the growth chamber under the same conditions. Mock inoculations were done with sterile distilled water only.

Sampling

Roots of *C. sativa* in contact with mycelium were recovered at different time points up to 48 h after contact. At each time point, 15 plants were randomly collected and grouped into three different pools (five plants each). Mock-inoculated controls were sampled simultaneously. The roots were immediately ground to a fine powder in liquid nitrogen and stored at −80°C until analysis.

Preparation of *C. sativa* roots for the scanning electron microscopy

Small pieces of well-washed *C. sativa* roots were collected after contact with *P. tinctorius* for 2, 12, or 24 h and fixed in 2.5% (v/v) glutaraldehyde (grade I, Sigma, St. Louis), 0.1-M Hepes buffer, and pH 6.8 for 24 h. After three washes in the same buffer, the samples were post-fixed in 2% (w/v) OsO_4 (Sigma) for 2 h at 4°C. The specimens were then washed in the same buffer, dehydrated in an ascending acetone series (10% steps, 30 min each), and dried in hexamethyldisilazan (Merck) for 1 min. Non-inoculated *C. sativa* roots were used as control. Specimens were mounted on aluminium stubs and coated with gold using a Fisons Instruments sputter coater SC502. The roots were then visualized in a Leica Cambridge scanning electron microscope, model S360, at 15 keV.

Quantification of hydrogen peroxide

Hydrogen peroxide content was determined according to Loreto and Velikova (2001). Briefly, samples (70 mg) were homogenized in 2.0 ml of 0.1% (w/v) trichloracetic acid, and the homogenate was centrifuged at 14,000×*g* for 15 min at 4°C. From each supernatant, an aliquot of 0.5 ml was added to 0.5 ml of 10-mM phosphate buffer (pH 7.0) and 1.0 ml of 1-M KI, and the absorbance was measured at 390 nm. H_2O_2 was quantified taking into account a calibration curve using solutions with known H_2O_2 concentrations.

Detection of superoxide production

Superoxide ($O_2^{\cdot-}$) production was examined in *C. sativa* roots up to 9 h during the time course of *P. tinctorius* contact, using a modification of the Nitro blue tetrazolium (NBT) staining technique described by Romero-Puertas et al. (2004). Root segments were incubated in 0.05% (w/v) NBT in 10-mM phosphate buffer (pH 7.5) at room temperature for 30 min. Bleaching was performed by the immersion of stained segments in boiling ethanol/lactic acid/glycerol (4:1:1) for 5 min. As control, non-inoculated *C. sativa* roots were stained using the same procedure. Segments were mounted on a glass slide in 60% (v/v) glycerol and examined with a Leitz Laborlux 12 microscope. The formation of a blue formazan precipitate indicates the reduction of NBT by superoxide ($O_2^{\cdot-}$).

Extraction and quantification of proteins

Approximately 1.0 g (FW) of ground root tissue was homogenized in 4 ml of 80-mM phosphate buffer (pH 7.0), 1-mM benzamidine, 0.1% (v/v) 2-mercaptoethanol, 1-mM EDTA, 0.1% (v/v) Triton X-100, and 1% (w/v) polyvinylpolypyrrolidone at 4°C. The homogenates were centrifuged at 14,000×*g* for 20 min at 4°C, and the supernatants were recovered. Protein was quantified by the Coomassie Blue microassay using Bovinum Serum Albumin (BSA) as standard (Sedmak and Grossberg 1977).

Enzymatic assays

All enzymatic assays were performed using freshly prepared protein extracts that were kept on ice until analysis.

The SOD (EC 1.15.1.1) activity determined according to the Beyer and Fridovich (1987) method was based on the ability of SOD to inhibit the reduction of NBT by the superoxide radicals generated photochemically. The reaction mixture consisted of 100-mM phosphate buffer (pH 7.8), 0.2-mM EDTA, 19.8-mM L-methionine, 0.05% (v/v) Triton X-100, 57-μM NBT, 0.9-μM riboflavin, and 50–250 μl protein extract. After 6 min of incubation at 30°C under continuous light, absorbance was read at 560 nm. One unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under the above assay conditions.

CAT (EC 1.11.1.6) activity was determined by following the decomposition of H_2O_2 (Aebi 1983). Reactions were performed in 80-mM phosphate buffer (pH 7.0) containing 1-mM H_2O_2 and initiated by adding 20–50 μl of protein extract. The decrease in absorbance was evaluated at 240 nm and 25°C. One unit (U) of CAT activity is defined as the amount of enzyme necessary to decompose 1 μmol min^{-1} H_2O_2 under the above assay conditions.

Evaluation of *P. tinctorius* growth in the presence of crude extracts prepared from elicited *C. sativa* roots with *P. tinctorius*

Crude extracts of *C. sativa* roots in contact with *P. tinctorius* mycelium up to 48 h were prepared, and the protein content was quantified as described previously. After filtration through a 0.45-μm filter, 100 μl of protein extract (0.25 μg μl^{-1} of protein) were plated onto 10 ml of MMN solid media at pH 6.6 in Petri dishes (9-cm diameter). The buffer used for protein extraction was used as control. Inoculation with *P. tinctorius* was performed with hyphal plugs (5-mm diameter; one plug per plate) collected at the margin of a 2-week-old culture. Five replicates for each time point were performed, and cultures were incubated at 25°C in the dark. Radial fungal growth was measured after 17 days of culture, and the results were expressed as millimetre per day.

Data analysis

Data from enzyme activities, quantification of H_2O_2, and radial growth of *P. tinctorius* are presented as the mean of three to five independent experiments with the respective SE bars. The differences between means were analysed by ANOVA using SAS v. 9.1.3, and averages were compared using the Tukey test. The significance was denoted by a p value less than 0.05.

Results

P. tinctorius adhesion to *C. sativa* roots

When root systems of 4-month-old seedlings of hydroponically grown *C. sativa* were inoculated with mycelia of *P. tinctorius*, early contact between symbionts was observed by scanning electron microscopy to check the

ability of *P. tinctorius* hyphae to adhere to the root surface (Fig. 1). Two hours after inoculation, adhesion of fungal hyphae to the host surface was observed, particularly to the surface of secondary roots (Fig. 1a). Afterwards, the density of mycelia progressively increased (Fig. 1b and c), resembling hyphal mantles observed in ECMs. For the studied period (2–24 h), the penetration of *P. tinctorius* hyphae into *C. sativa* root cells were not observed (Fig. 1d and e).

Quantification of ROS

To ascertain the involvement of ROS during the early stages of *C. sativa–P. tinctorius* contact, the quantification of hydrogen peroxide and detection of superoxide anion was performed. As depicted in Fig. 2a, three peaks of H_2O_2 production were observed. The first peak occurred 2 h after *P. tinctorius* inoculation, exhibiting a significant increase (up to 1.5-fold) when compared with the corresponding

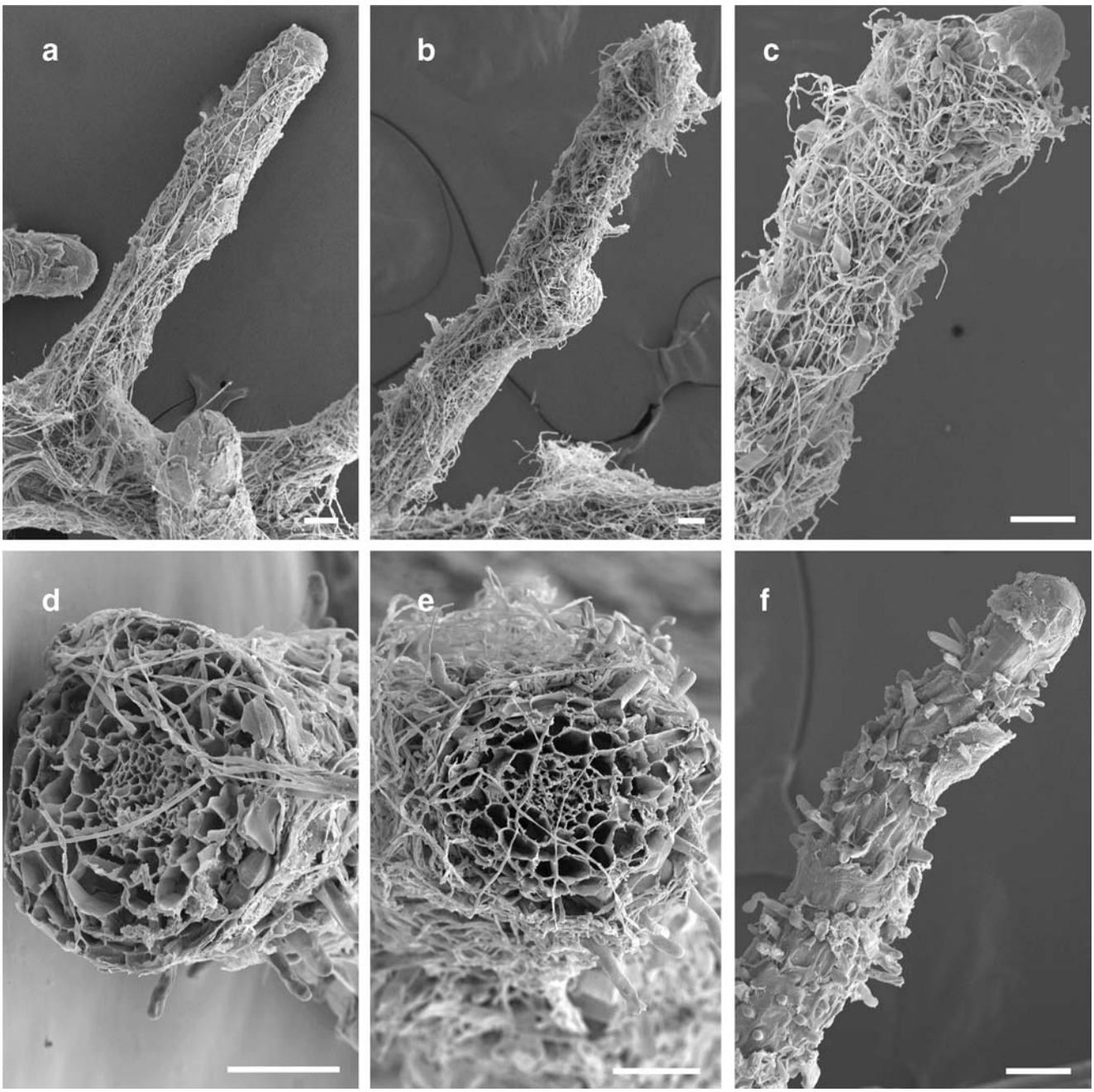

Fig. 1 Scanning electron micrographs of *Castanea sativa* roots after contact with *Pisolithus tinctorius* for 2 h (**a**), 12 h (**b**), and 24 h (**c**). Root cross-sections 2 h (**d**) and 24 h (**e**) after fungal inoculation are also displayed. Non-inoculated plants were used as control (**f**). Bars=50 μm

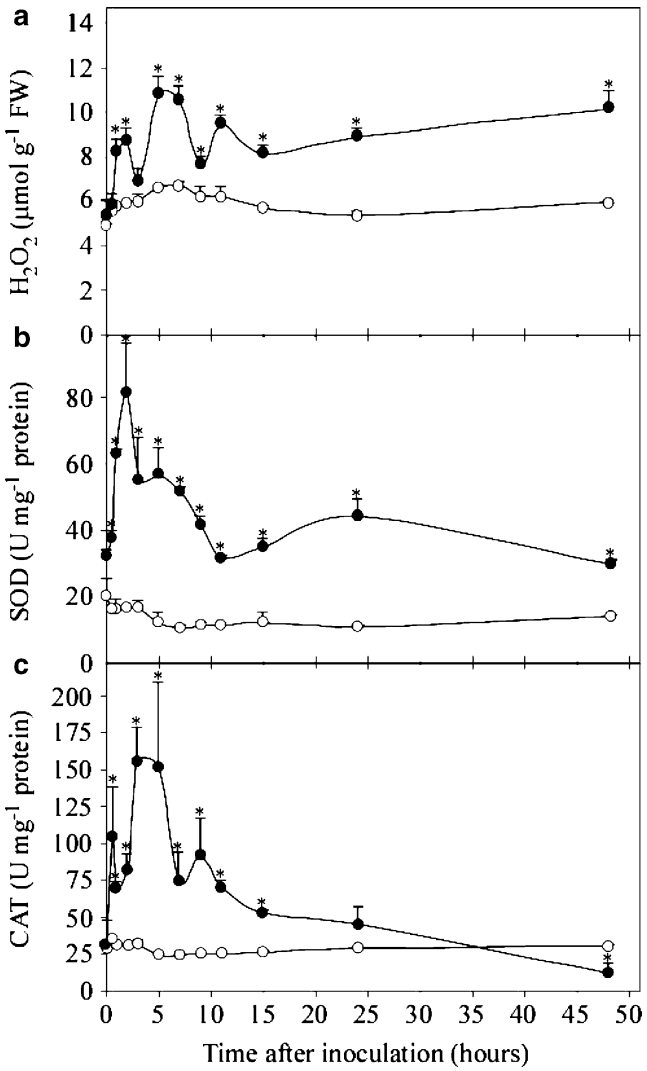

Fig. 2 The quantification of H_2O_2 levels (**a**) and the determination of SOD (**b**) and CAT (**c**) activities in *Castanea sativa* roots after the inoculation with *Pisolithus tinctorius* (*closed circles*). Mock-inoculated plants (*open circles*) were used as controls. Mean±SE (n=3 or 9) is shown. Statistically significant differences between inoculated and mock-inoculated (control) plants are indicated with an *asterisk*; $p<0.05$

control. Two additional peaks, 5 h (6.3-fold increase) and 11 h (1.5-fold increase) after the *P. tinctorius* inoculation were also observed. After a slight decrease in H_2O_2 levels at 15 h after inoculation, a statistically significant gradual increase was observed up to 48 h after inoculation, relative to mock-inoculated roots.

The production of superoxide anion in the roots of *C. sativa* elicited by *P. tinctorius* was evaluated by observing the insoluble blue-coloured formazan complex indicative of NBT reduction by $O_2^{\cdot-}$. Results (Fig. 3) revealed that the fungus was able to induce the production of superoxide anions in *C. sativa* roots, mainly in the epidermal cells of the secondary roots. In non-elicited *C. sativa* roots, no accumulation of formazan complexes was observed. The accumulation of $O_2^{\cdot-}$ was time-dependent, with the identification of two distinct bursts. NBT reduction was evident 0.5 h after fungal contact with the accumulation of insoluble formazan dispersed in specific root regions at the cell surface. After 2–3 h of fungal contact, NBT reduction was not observed. A second burst of $O_2^{\cdot-}$ production was detected after 5 to 7 h of fungal contact. In contrast to what was observed for the first $O_2^{\cdot-}$ burst, the accumulation of insoluble formazan occurred intracellularly. This transient $O_2^{\cdot-}$ accumulation disappeared after 9 h of fungal contact.

Activity of ROS-scavenging enzymes

SOD and CAT activities were determined in protein extracts from *C. sativa* roots during the time course of elicitation by *P. tinctorius* mycelia. For all sampling times, SOD activity was significantly higher in inoculated plants than in non-inoculated controls (Fig. 2b). In addition, three peaks of SOD activity were observed. The first peak, corresponding to the highest SOD activity value detected (up to 4.9-fold), was observed 2 h after inoculation. The second (4.6-fold) and third (4.0-fold) peaks were detected 5 and 24 h after fungal contact, respectively.

CAT activity was also assayed in protein extracts prepared from *C. sativa* roots up to 48 h after inoculation with *P. tinctorius* (Fig. 2c). A three-fold significant increase in CAT activity was observed 0.5 h after elicitation, when compared to the corresponding control. A second (6.3-fold) and a third (3.6-fold) significant increase in CAT activity were also detected 3–5 and 9 h after *P- tinctorius* inoculation, respectively.

P. tinctorius growth in the presence of crude extracts from *P. tinctorius*-elicited *C. sativa* roots

The effect of components produced up to 48 h after inoculation by the association of *C. sativa* roots with *P. tinctorius* on the growth of *P. tinctorius* hyphae was evaluated. Radial mycelium growth was measured after 17 days of culture on solid MMN medium containing crude aqueous root extracts. In the presence of root extracts, fungal growth was stimulated as compared to the growth of the control samples (solid medium containing extraction buffer; Fig. 4). Fungal growth stimulation was evident by root extracts made immediately after inoculation (0 h sampling), and varied with time after inoculation. Stimulation in *P. tinctorius* growth was observed in the presence of root extracts prepared 0–2, 7, 11, and 24 h after inoculation. In contrast, the stimulation of fungal growth was constrained by the extracts of root inoculated with *P. tinctorius* for 3, 9, and 15 h. No morphological differences (such as mycelium texture, colour, morphology, or medium

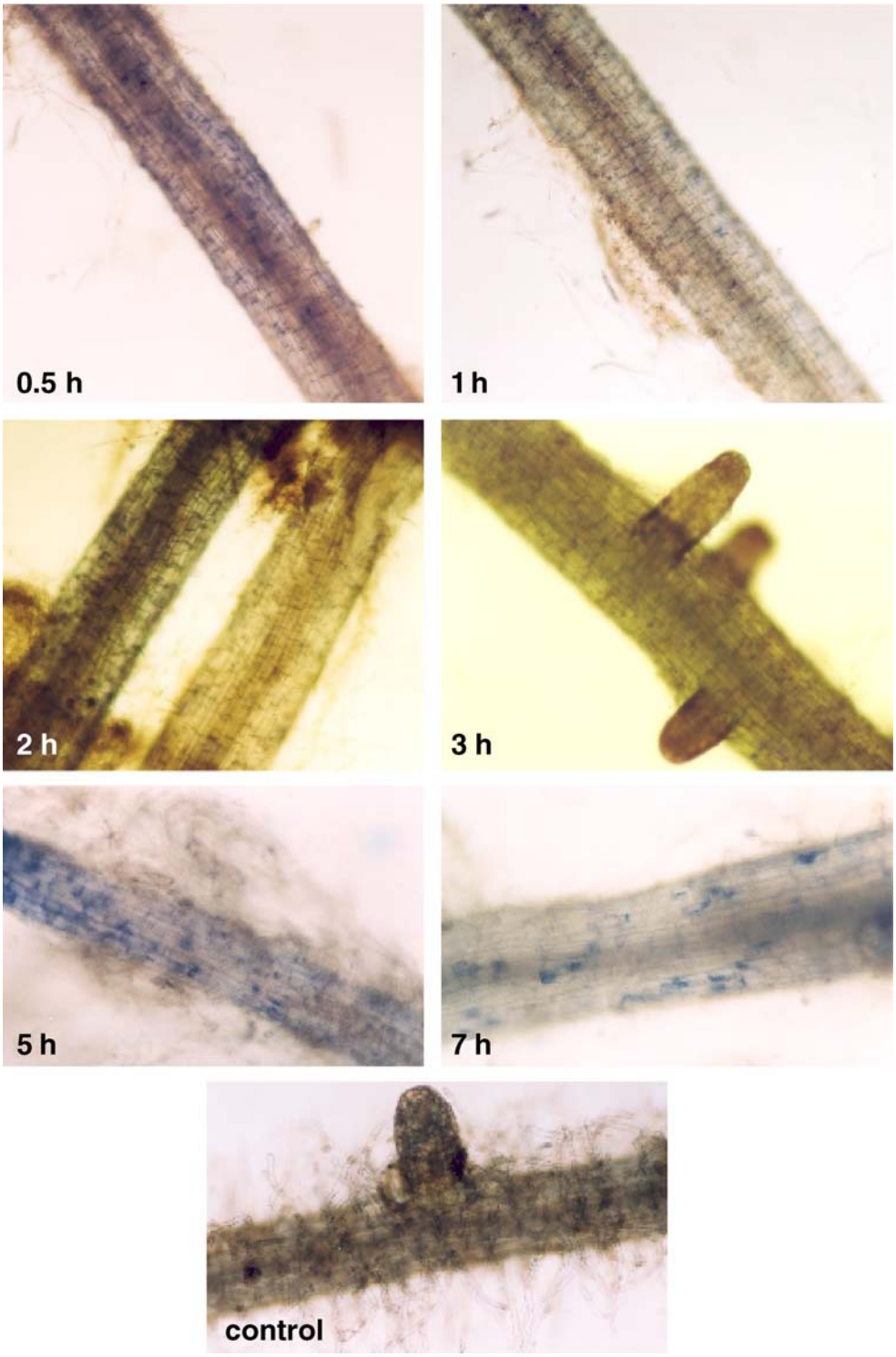

Fig. 3 Localization of superoxide anions by NBT staining in *Castanea sativa* roots after contact with *Pisolithus tinctorius*. Staining was performed during the time course of fungal elicitation (0.5–7 h). Superoxide production was detected by the accumulation of blue formazan precipitates. Mock-inoculated plants were used as controls. Magnification ×100

colouration) were found between *P. tinctorius* grown in the presence of extracts from inoculated or control roots.

Discussion

The development of ECM symbioses requires a series of complex and overlapping ontogenic processes in the symbionts. These include hyphal attachment to host roots, hyphal aggregation around the host root to form a multicellular structure—the mantle—and hyphae penetration between the root cells to form the Hartig net (Martin et al. 1999, 2001). In the hydroponic system used in this work, contact was observed between *P. tinctorius* and *C. sativa* roots 2 h after inoculation. Because wall-to-wall contact has been suggested to be crucial to the signalling

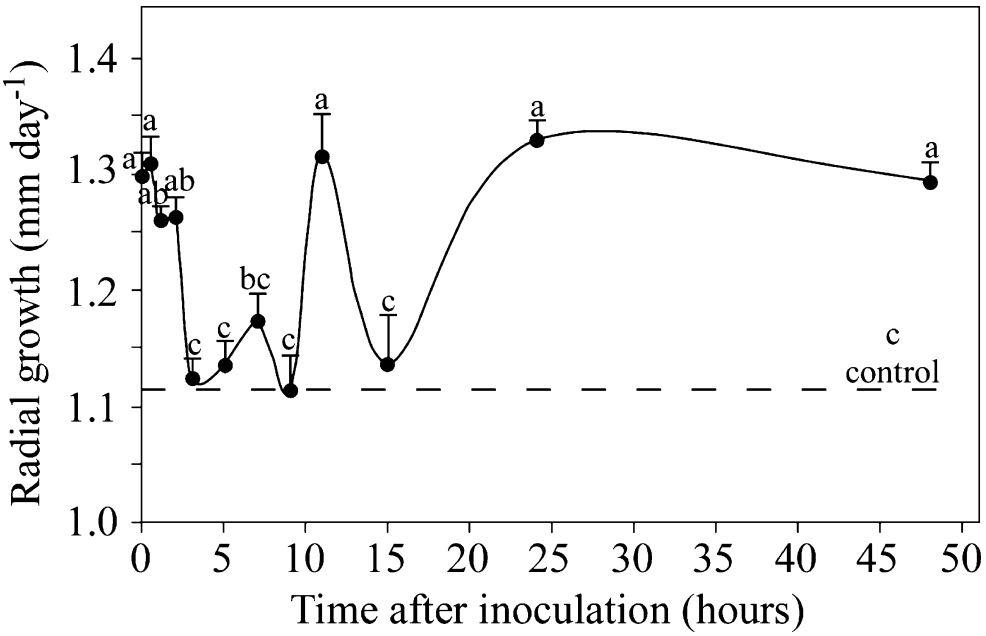

Fig. 4 Radial growth of *Pisolithus tinctorius* hyphae after 17 days of culture on agar MMN media in the presence of protein extracts taken from *C. sativa* roots inoculated with *P. tinctorius* (from 0 to 48 h). As a control, agar media were supplemented with protein extraction buffer. Mean±SE (n=5) is shown. The *letters above the columns* indicate significant difference at p<0.001

process in ECM (reviewed by Sirrenberg et al. 1995), this observation indicates that the in vitro system used in the present work is suitable to study events related to the generation of signals between the symbionts. At 12 h after inoculation, *P. tinctorius* hyphae were densely disposed around *C. sativa* roots, resulting in a multicellular structure that could correspond to an incipient mantle. Up to 24 h after elicitation, hyphal penetration of roots was still not evident.

An oxidative burst is generally defined as a rapid production of high amounts of ROS in response to external stimuli and has been considered as one of the earliest events of plants to pathogenic microorganisms (Lamb and Dixon 1997; Wojtaszek 1997). Initial evidence for the accumulation of ROS in arbuscular mycorrhiza was reported by Salzer et al. (1999) for the *Medicago truncatula–Glomus intraradices* association. Since then, other examples have been reported (García-Garrido and Ocampo 2002). In ECM associations, the evidence for the involvement of ROS accumulation during mycorrhiza establishment is lacking. To evaluate the role of ROS in ECM establishment, we focussed on ROS production during the early stages of the symbiotic interaction (0–48 h) between *C. sativa* and *P. tinctorius*. After being kept in contact with *P. tinctorius*, *C. sativa* roots were extensively washed from mycelia and analysed for H_2O_2 content. Three peaks of H_2O_2 production were detected, corresponding to a pattern similar to what has been observed for pathogenic infections, although differences in the times of ROS production have been reported for distinct biological species (Wojtaszek 1997). In incompatible plant–pathogen interactions, oxidative bursts generally comprise a first weak transient peak 1–2 h after infection (phase 1) followed by another peak of a longer and greater magnitude 3–6 h after infection (phase 2; Lamb and Dixon 1997; Mahalingam and Fedoroff 2003). Because phase 2 is only triggered by avirulent pathogens, the weak transient phase 1 burst seems to be a biologically non-specific reaction, whereas the phase 2 burst has been reported to be involved in plant HR associated with a localized cell death. Although early contact between *P. tinctorius* and *C. sativa* roots produced oxidative bursts, the massive root colonization by the mycobiont does not induce cell death at the infection site as in a typical HR, suggesting that ECM fungi are able to circumvent the common host defence responses facilitating fungal invasion.

Most of the available data obtained from plants challenged with pathogens including fungi, bacteria, and viruses, as well as from cultured cells treated with elicitors, implicate H_2O_2 as the major ROS produced during the oxidative burst (Wojtaszek 1997). However, in some cases, it becomes difficult to clearly identify the ROS behind the oxidative burst due to the inherent relationship between H_2O_2 and $O_2^{\cdot-}$ generation. In the present work, evidence for the capacity of *P. tinctorius* fungi to induce the production of ROS in *C. sativa* roots is provided. Two $O_2^{\cdot-}$ bursts were detected by formazan blue accumulation during the time course of *P. tinctorius* development on *C. sativa* roots, and these were coincident with the two first H_2O_2 production peaks. The absence of $O_2^{\cdot-}$ staining in hyphae indicates that ROS production is only taking place in plant tissue. In the first phase of $O_2^{\cdot-}$ production, this ROS appeared to be produced extracellularly, suggesting that the early reaction to the ECM fungus occurred at the cell wall and plasma membrane levels. This is a common feature of the pathogenic attack responses (Wojtaszek 1997) where potential sources of ROS production have been mainly ascribed to plasma membrane-bound nicotinamide adenine

dinucleotide phosphate oxidases, cell-wall-bound POXs and amine oxidases (reviewed by Mittler 2002). During the second phase of ROS production, in the *P. tinctorius–C. sativa* interaction, it was possible to identify microdomains of $O_2^{\cdot-}$ accumulation within cells that could result from the activation of ROS-generating systems and the down-regulation of ROS-scavenging systems. The decline in cell capacities to scavenge ROS by suppressing ROS-scavenging systems has been thought to account for the over-accumulation of ROS (reviewed by Mittler 2002).

The efficient dismutation of $O_2^{\cdot-}$ and H_2O_2 decomposition requires a synchronized action of several antioxidant enzymes. SOD dismutates superoxide to H_2O_2; however, it only converts one destructive ROS to another, whereas CAT converts H_2O_2 to non-ROS. Although a coordinated action between SOD and CAT could attenuate ROS accumulation, the imbalanced activities of both enzymes could also enhance ROS production. In the *C. sativa–P. tinctorius* system, a rapid increase in SOD activity was coincident with the first H_2O_2 peak that occurred 2 h after the *P. tinctorius* inoculation. These data suggest that early increases in SOD activity could contribute to the observed high levels of H_2O_2. The involvement of SOD in ECM association has also been suggested to be responsible for the increase in H_2O_2 in suspended spruce cells incubated with elicitors from the ECM fungi *A. muscaria* or *H. crustuliniforme* (Schwacke and Hager 1992). Additionally, the increase in SOD activity could be one strategy to cope with excess superoxide radicals generated during the early stages of symbiosis establishment.

Coincident with an increase in SOD activity observed 2 h after *P. tinctorius* inoculation, the observed decline in CAT activity could account for H_2O_2 overproduction. The subsequent increase in CAT activity 3–5 h after *P. tinctorius* inoculation could reflect the ability of H_2O_2 scavenging in the system. These results are in accordance with those obtained from the elicitation of suspended spruce cells by cell wall fragments of the ECM fungi *H. crustuliniforme* and *A. muscaria*, further supporting the hypothesis that CAT is involved in the regulation of ROS production during ECM establishment (Schwacke and Hager 1992). The temporal activation of CAT may be important in the protection of host plant roots against the overproduction of ROS that could otherwise induce cell death. Decreased CAT activity, observed between 15–48 h after inoculation with *P. tinctorius*, may be sufficient to achieve optimal H_2O_2 levels (significantly higher than the control for this period of time) to ensure signal transduction whilst avoiding uncontrolled oxidative damage. Therefore, the combined action of SOD and CAT in detoxifying superoxide radicals and hydrogen peroxide, respectively, could prevent host cell damage during ECM establishment.

Compounds identified in root exudates and extracts have been described as affecting the chemical and physical environment of the rhizosphere and the establishment of root-microbe communication (reviewed by Hirsch et al. 2003; Bais et al. 2004). To investigate whether such phenomena may be actuate in *P. tinctorius–C. sativa* interactions, the growth of *P. tinctorius* hyphae was measured in the presence of crude extracts from *P. tinctorius*-inoculated *C. sativa* roots. The stimulation of fungal growth was observed in the presence of root extracts, which agrees with previously reported increased hyphal growth in response to host root exudates and root extracts in both ECM and AM fungi (Sirrenberg et al. 1995; Giovannetti and Sbrana 1998; Lagrange et al. 2001; Lum and Hirsch 2003). Amongst the compounds released in root exudates, rutin (Lagrange et al. 2001), isoflavonoids, and flavonoids (Kapulnik et al. 1996; Lum and Hirsch 2003) have been suggested to be involved in the stimulation of hyphal growth. However, the stimulating activity of root extracts decreased after 3, 9, and 15 h of root inoculation with *P. tinctorius*, and this superimposed decreases in H_2O_2 production. These results suggest that the two phenomena may somehow be related. The accumulation of H_2O_2 seems to result from a regulated time-dependent stimulation of ROS generating systems and decrease of ROS-scavenging enzyme activities. This ROS may be an important initial product for regulating interactions during the early stages of ECM establishment and a candidate signalling molecule for symbiosis development. Further experiments are needed to confirm this hypothesis and to address changes that may occur with other signalling molecules.

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